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(54) Title: OLIGODENDROCYTE CELL CULTURES AND METHODS FOR THEIR PREPARATION AND USE

(57) Abstract: An *in vitro* differentiated oligodendrocyte-enriched cell culture is disclosed. Methods of making the cell culture are also disclosed. Methods are described for using *in-vitro* differentiated neural cells, preferably enriched in oligodendrocytes, in transplantations to treat trauma or degeneration of the spinal cord.

OLIGODENDROCYTE CELL CULTURES AND METHODS FOR THEIR PREPARATION AND USE

This application claims priority to U.S. Provisional Application No. 60/161,125, filed October 22, 1999, the entirety of which is incorporated by reference herein.

Pursuant to 35 U.S.C. §202(c), it is acknowledged that the U.S. Government has certain rights in the invention described herein, which was made in part with funds from National Institute of Health Grant Numbers NINDS, NS01931, NS36265 and NS37927.

FIELD OF THE INVENTION

This invention relates to the field of cell culture methods and methods for treatment of diseases of the central nervous system.

BACKGROUND OF THE INVENTION

Various scientific and scholarly articles are referred to throughout the specification. These articles are incorporated by reference herein to describe the state of the art to which this invention pertains.

Recovery in central nervous system (CNS) disorders is hindered by the limited ability of the vertebrate CNS to regenerate lost cells and re-establish functional connections. In many CNS disorders, including multiple sclerosis, stroke, spinal cord injury and other trauma, demyelination of intact axons is an important factor contributing to loss of function. Previous studies suggest that substantial recovery of function might be achieved through remyelination of otherwise intact neural pathways. As a therapeutic modality, functional recovery through remyelination may prove much easier to achieve than recovery via regeneration of severed axons, where appropriate synaptic connectivity must also be re-created.

Transplantation approaches utilizing cellular bridges, fetal CNS cells, fibroblasts expressing NT-3, hybridoma cells expressing inhibitory protein blocking antibodies, or olfactory ensheathing glial cells into the acutely injured spinal cord has

produced axonal regrowth or functional benefits. Transplants of rat or cat fetal spinal cord tissue into the chronically injured cord survive and integrate with the host cord, and may be associated with some functional improvements. In addition, rats transplanted with fetal spinal cord cells exhibited benefits in some gait parameters, and the delayed transplantation of fetal raphe cells can enhance reflexes. Neural progenitors isolated from the adult CNS differentiate into neurons and glia after transplantation into brain (Gage, et al., (1995) Proc. Natl. Acad. Sci. 92, 11879-11883), and differentiate into oligodendrocytes and astrocytes after transplantation into spinal cord.

Neural transplantation studies have been limited by ethical considerations and a lack of a reliable source for undifferentiated pluripotent cells. *In vivo* differentiated neural cells cultures are problematic because may contain genetically abnormal cells. Ideally, *in vitro* neural cell cultures would provide a better source of materials for transplantation, and other commercial and research purposes.

ES cells provide a partial solution to the problems encountered with *in vivo* derived neural cells because they are genetically normal, totipotent, capable of indefinite replication (Suda, Y et al, (1987) J. Cell. Physiol. 133, 197-201) and have been derived from several vertebrate species including mice (Evans & Kaufman, (1981) Nature 292, 154-156; Martin, (1981) Proc. Natl. Acad. Sci. USA 78, 7634-7638) and humans (Thomson, et al., (1998) Science 282, 1145-1147; Shamblott, et al., (1998) Proc. Natl. Acad. Sci. USA 95, 13726-13731). ES cells are also among the most flexible stem cell for genetic engineering. For example, the production of double gene allele knockouts in single ES cells has been accomplished (Wilder, et al., (1997) Dev. Biol. 192, 614-629; Hakem, et al., (1998) Cell 94, 339-352).

In theory, ES cells can generate all cell types and preliminary work has shown that differentiation into oligodendrocytes is possible (Fraichard, et al., (1995) J. Cell Sci. 108, 3181-3188; Dinsmore, et al., (1996) Cell Transplant. 5, 131-143; Brustle, et al., (1997) Proc. Natl. Acad. Sci. USA 94, 14809-14814; Brustle, et al., (1999) Science 285, 754-756). However, simple and reliable methods for producing and enriching oligodendrocytes have not been developed.

SUMMARY OF THE INVENTION

The present invention provides a method to treat spinal cord degeneration through the transplantation of *in vitro* differentiated cultures of neural cells. The inventors, through their superior appreciation of the problems of spinal cord transplantation techniques, have recognized the advantages of *in vitro* cultured cells. Furthermore, the inventors have discovered that oligodendrocytes are the critical cells required for transplantation. In accordance with the discovery of the importance of oligodendrocytes, a method to make an enriched culture of oligodendrocytes is provided. The highly enriched oligodendrocyte culture of the invention can be used to particular advantage in the method to treat spinal cord degeneration of the invention to increase the myelination of axons in degenerated spinal cord tissues.

According to one aspect of the present invention, a method to make a cell culture highly enriched in oligodendrocytes is provided. In one preferred embodiment, the method uses retinoic acid differentiated 4-/4+ stage EB cells. In another preferred embodiment, the method uses preconditioned oligosphere media, thereby forming an intermediate cell type referred to herein as "oligospheres".

Another aspect of the invention features a cell culture enriched in oligodendrocytes. The oligodendrocyte-enriched culture may comprise from about 20% to about 99% oligodendrocytes in the population. In a preferred embodiment, the culture is made by the inventive method described herein.

According to another aspect of the invention, a method is provided for using an *in vitro* differentiated culture of neural cells to treat degenerated CNS tissue. This method comprises the steps of transplanting *in vitro* differentiated neural cells into the spinal cord of a patient in need of such treatment, and allowing the transplanted cells to replace damaged or missing tissues. In a preferred embodiment, the neural cell culture of this method comprises the enriched oligodendrocyte culture of the invention.

Other features and advantages of the present invention will be understood by reference to the drawings, detailed description and examples that follow.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. BrdU labeled ES cell-derived cells 2 weeks after transplantation. Mean±SEM BrdU labeled nuclei per one mm segment in longitudinal sections (n = 11 rats, 3 sections per rat).

Fig. 2. ES cell-derived cell transplantation improved behavioral recovery. **Fig. 2a)** Closed circles, ES cell transplant group; open circles, vehicle treated group (n=11 per group, mean ± SEM). *Difference at $P < 0.05$ vs. control at same time point (repeated measures ANOVA with Tukey's test). **Fig. 2b)** Similar experiment comparing transplantation of ES cells (closed circles), vehicle (open circles), or adult mouse neocortical cells (closed diamonds) (n = 6 per group). The ES cell transplantation group differed from both control groups at the $P<0.05$ level. Arrows indicate transplantation.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides compositions and methods to regenerate the tissue of the central nervous system. Surprisingly, it has been discovered that the transplantation of a mixed population of *in vitro* differentiated neural cells into a damaged spinal cord is associated with improvements of CNS function. It has further been discovered that a preparation of a highly enriched culture of *in vitro* differentiated oligodendrocytes is unexpectedly effective in restoring CNS function by transplantation.

Thus, in accordance with the invention, a method to make a highly enriched culture of oligodendrocytes is provided. These highly enriched cultures of oligodendrocytes can be produced from ES or other totipotent cells by generating an intermediate stage of floating cell groups termed "oligospheres". Oligospheres primarily contain an early form of oligodendrocyte progenitor, and can be produced by culturing a mixed population of *in vitro* differentiated neural cells in a preconditioned oligosphere medium, described in greater detail below. The resulting highly enriched culture of oligodendrocytes is a novel cell culture that will have many uses in research as well as transplantation therapy.

As described in Example 1, mouse derived embryonic stem (ES) cells induced to differentiate into a mixed population of neural cells by retinoic acid were transplanted into physically and chemically injured spinal cords of rats. The transplanted mouse cells differentiated into primarily oligodendrocytes in the spinal tissue and the rats had improved hind-limb functionality as compared to the control group. The use of ES cultures was surprisingly superior to the use of *in vivo* derived cells in the speed at which the transplanted cells migrated into the damaged spinal cord tissue and degree of remyelination.

A method to culture a highly enriched population of oligodendrocytes from mixed population of neural cells was developed and optimized, as described in Examples 2 and 3. The method utilizes a novel culture medium that selectively encouraged the growth of an intermediate class of cells termed "oligospheres", containing primarily immature and mature oligodendrocytes. This enrichment method is based on the unexpected simple concept of using the preconditioned media from existing oligodendrocyte cultures to selectively induce the division of oligodendrocytes in a mixed population to neural cells.

When this enriched oligodendrocyte culture was transplanted into the spinal cord of *shiverer* mice, which lack myelin basic protein, the introduced cells oriented with native oligodendrocytes and myelinated the native axons. The enriched oligodendrocyte culture was surprisingly more effective than the retinoic acid differentiated ES cells in the rate of cell migration and myelination in the spinal cord.

The present invention provides a method of therapy for degenerated CNS tissues. The method comprises the step of transplanting *in vitro* differentiated neural cells into the site of injury and allowing sufficient time for the introduced cells to replace missing or defective cell types. This method is particularly effective when a highly enriched population of oligodendrocytes is used for the transplantation.

While not limiting the way in which the transplanted cells benefit CNS function to any one theory, it appears that the transplanted oligodendrocytes act by remyelinating damaged axons. In many injuries to the spinal cord, the axons remain intact but are rendered useless due to the loss of their myelin sheath. Oligodendrocytes in the CNS (and Schwann cells in the peripheral nervous system)

wrap the axon of a nerve in a myelin sheath and prevent current from leaking across the axonal membrane. The insulating function of the myelin allows the action potential to move faster along the axon and to use less metabolic energy. When the myelin sheath is absent from the majority of axons in a region due to injury, genetic mutation or disease, signals cannot be carried up and down the spinal cord, resulting in loss of muscular control and/or sensory signals. Remyelination may therefore be able to restore nervous system function in some situations.

Accordingly, the use of *in vitro* differentiated oligodendrocytes is particularly useful in situations involving loss of myelination. In particular, this method of therapy is contemplated to be useful for treating multiple sclerosis, Alzheimer's Disease, leukodystrophies, cerebrial palsey, stroke, cardiac arrest and CNS trauma, among others.

To facilitate performance of the aforementioned therapeutic methods, the inventors have determined how to manipulate cultured pluripotent cells to produce a culture of differentiated neural cells that is highly enriched in oligodendrocytes. Accordingly, an oligodendrocyte-enriched cell culture is featured in accordance with the present invention. It has been discovered that such an oligodendrocyte-enrichced culture comprising as few as about 20% oligodendrocytes in the population is suitable for use in the therapeutic methods of the invention. However, the inventors have devised methods for producing a culture comprising up to 99% oligodendrocytes. Therefore, the present invention includes oligodendrocyte-enriched cultures comprising at least about 20%, preferably at least about 30%, more preferably at least about 40%, yet more preferably at least about 50%, and even more preferably at least about 60%, 70%, 80% or 90%, oligodendrocytes in the population.

Oligodendrocyte-enriched cultures derived from ES cells are exemplified in the present invention. However, other pluripotent cell types can be differentiated into neural cells; therefore, oligodendrocyte-enriched cultures derived from any pleuripotent cell type are included in the invention. These include, but are not limited to, progenitor cells from the developing nervous system, cells derived from naturally occuring carcinomas and embryonal carcinoma cells, such as P19 (Bain et al., 1998), among others.

Since basic features of pleuripotent cells are consistent across species, oligodendrocyte-enriched cell cultures from such cells of any vertebrate are included in the invention. In a preferred embodiment, the oligodendrocytes are produced from mammalian cells; particularly preferred are primate cells, and most preferred are human cells. Other vertebrate cells from which oligodendrocyte-enriched cultures can be obtained include, but are not limited to, laboratory rodents such as mice (exemplified herein), rats (also exemplified herein) and hamsters, and domesticated animals such as cats, dogs and horses.

Presented with the present invention is a method to make a highly purified culture of oligodendrocytes. This method comprises the several steps of:

1. typsinizing and triturating mixed cultures of oligodendrocytes, neurons, and astrocytes from 4-/4+ stage embryoid bodies (to dissociate the cells);
2. culturing the cells in culture flasks with modified SATO medium (as described below) for a specified period (e.g., 3 days *in vitro* (DIV));
3. gently shaking the flasks to suspend loosely adhering cells (primarily oligodendrocytes) while astrocytes remain adhering to the flasks; and
4. transferring the suspended cells to a new flask containing modified SATO medium at a 1:1 ratio for an additional culture period.

Example 3 sets forth a detailed set of instructions on how to prepare an oligodendrocyte-enriched culture from ES cells.

This last step, in which the cells are further cultured in a medium that contains both fresh SATO medium and "old" medium that is oligsphere-conditioned,is key to culturing this highly enriched culture. While not limiting the operation of this culture method to any one explanation, it is likely that differentiated oligodendrocytes condition the medium in which they grow with a factor(s) that selectively promotes the survival and proliferation of oligodendrocytes. The culture method produces oligospheres, which are primarily composed of immature and mature oligodendrocytes and nestin positive progenitor cells.

This novel oligodendrocyte culture method has several advantages over previous methods. The few astrocytes that are generated in this method adhere to the side of the culture flask and are easily removed from the culture. Additionally, the

free-floating spherical cell clusters (oligospheres) can be easily concentrated or moved to a different medium for use.

The method to produce an enriched oligodendrocyte culture may be used with EB cells from other species. Examples 1 and 2 teach the use of the method with *Mus musculus* and *Rattus* EB cells, illustrating the flexibility of the method. Due to the great similarity between EB cells from all mammalian species, it is contemplated that the culture method may be used with equal efficacy with EB cells of other species. Species of particular interest include, but are not limited to, human and monkey.

Bioengineered stem cell cultures may also be used to advantage with the culture method. Autologous ES cells are particularly advantageous when the cells are to be used for transplantation. Autologous ES cells may be created by transferring a ES cell nucleus into a denucleated cell from the patient. These autologous ES cells will have the totipotent characteristics of the ES cell but will not be rejected when transplanted into the patient. Embryonic stem cell cultures from the same species may also be genetically engineered to remove markers that will cause rejection in the patient.

Also presented with the invention is a method to regenerate the central nervous system in patients that are deficient in axon myelination, which encompasses introducing oligodendrocyte cells cultured *in vitro* from embryonic stem cells. The use of *in vitro* cultures is superior to previous methods of transplanting *in vivo* differentiated cells because it is known that the ES cells are genetically normal while *in vivo* differentiated populations may be harboring tumor cells. The use of ES-derived oligodendrocytes is also less invasive than previous methods where precursor cerebellar cells were used.

Embryonic stem cells are also advantageous over *in vitro* differentiated cells because they can be easily genetically modified. The ES cells may be engineered to produce factors that will aid spinal cord regeneration and/or make the cell more likely to survive transplantation, i.e. tolerance of low oxygen conditions. Factors of particular interest for spinal cord reneration include, but are not limited to, neurotrophin 3 (NT-3), β-FGF and L1. The use of embryonic stem cells to culture an

oligodendrocyte culture is particularly advantageous because it allows cells that are genetically identical to the patient to be treated.

While the use of enriched cultures of oligodendrocytes for transplantation therapy is discussed above, this enriched cell culture is also extremely valuable for research purposes. The oligodendrocyte culture of the invention may be used to study disorders of oligodendrocyte cells found in humans, the mechanisms that regulate production of oligodendrocytes and the factors that the cells produce to regulate the development of other oligodendrocytes. This culture may also be used to develop an *in vitro* model system of neural tissue that will be invaluable for understanding how such tissues work and can be regenerated.

The following examples are provided to describe the invention in greater detail. They are intended to illustrate, not to limit, the invention.

EXAMPLE 1
Preparation and Use of *In Vitro* Differentiated Neural Cells to Regenerate the Central Nervous System

METHODS

Cell culture. D3 or ROSA26 mouse ES cells were maintained and differentiated in culture according to the 4-/4+ protocol of Bain et al. (Dev. Biol. 168, 342-357 (1995)). Undifferentiated ES cells were propagated in the presence of leukemia inhibitory factor (LIF, Gibco). Cells were cultured as embryoid bodies in the absence of LIF for 4 days, then treated for 4 days with retinoic acid (all-*trans*-RA, 500 nM, Sigma). On the 9th day, embryoid bodies were partially trypsinized (5 min. at 37°C, 0.25% trypsin with EDTA) and resuspended in ES cell media (Bain, et al., Dev. Biol. 168, 342-357 (1995)) prior to transplantation.

Spinal cord injury. Impact injury was induced using the weight drop device developed at New York University as described previously (Basso, et al., J. Neurotrauma 12, 1-21 (1995); Liu, et al., J. Neurosci. 17, 5395-5406 (1997)). Adult Long Evans female rats (275 ±25 g; Simonsen Lab, Gilroy, CA) were anesthetized with pentobarbital (50 mg/kg, i.p.), a laminectomy performed at T9-10 level, and the

dorsal surface of the cord subjected to weight drop impact, using a 10 gm weight (2.5 mm diameter) dropped at a height of 25 mm (Liu, et al., J. Neurosci. 17, 5395-5406 (1997)). During surgery, the rectal temperature was maintained at 37.0 ± 0.5 °C by a thermostatically-regulated heating pad (Versa -Therm 2156, Cole-Parmer, Chicago, IL), and during recovery, rats were placed in a temperature- and humidity-controlled chamber (Thermocare Inc., Incline Village, NV) overnight.

Transplantation. BBB scores were obtained the day before transplantation (day 8 post-injury) and control and experimental groups were matched and randomly assigned to ensure that initial locomotor scores were equalized between groups. The weight-drop injury level was chosen based on previous experience with the NYU impact model, to produce spontaneous recovery at a BBB score 8, the most sensitive portion of the scale corresponding to absent weight supported walking. Nine days after impact injury, rats received transplants of neural differentiated ES cells (approximately 1 million), vehicle medium, or 1 million adult mouse neocortical cells using a spinal stereotaxic frame, a 100 μm diameter tip glass pipette configured to a 5 μl Hamilton syringe, and a Kopf microstereotaxic injection system (Kopf Model 5000 & 900). Five μl of the ES cell or mouse neocortical cell suspension or vehicle medium was injected into the center of the syrinx at the T9 level over a 5 minute period. Three independent experiments with time matched controls were completed in total. The first series was completed for behavioral analysis and late histologic analysis [5 weeks post-transplantation, n = 11 per group, ES cell transplantation vs. vehicle medium control, D3 ES line used]. The second series was used to compare early (2 weeks post-transplantation) and late (5 weeks post-transplantation) histological outcomes [n = 11 per group, ES cell transplantation (ROSA-*lac-Z* transgene line) vs. vehicle medium control]. In the third series, 3 groups were compared for behavioral outcome to assess the effects of rat immune reactions to mouse cells [n = 6 per group, neural differentiated ES cell transplantation (ROSA26 *lac-Z* transgene line) vs. mouse neocortical cell transplantation vs. vehicle medium control, survival to 5 weeks post transplantation]. All groups received the same cyclosporine immunosuppression.

Animal care. All surgical interventions and animal care were

provided in accordance with the Laboratory Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals (NIH, EHEW Pub. No. 78-23, Revised, 1978) and the Guidelines and Policies for Rodent Survival Surgery provided by the Animal Studies Committee of Washington University School of Medicine. Manual bladder expression was performed 3 times daily until reflex bladder emptying was established. Cyclosporine (10 mg/kg, s.q.) was administered daily to all animals in every group beginning the day of transplantation.

Behavioral testing. Behavioral testing was performed weekly by two individuals blinded to treatment using the BBB Locomotor Rating Scale (Basso, et al., J. Neurotrauma 12,1-21 (1995)). Behavioral outcomes and examples of specific BBB locomotor scores were recorded using digital video.

Immunohistochemistry. Primary antibodies used were directed against the following antigens: astrocytes, GFAP rabbit polyclonal, 1:4 (Incstar); oligodendrocytes, APC CC-1 mIgG$_1$, 1:400 (Calbiochem Oncogene Sciences); neurons, NeuN mIgG$_1$, 1:500 (Chemicon); anti-mouse EMA rat hybridoma, 1:1 (Baumrind, et al., Dev. Dyn. 194, 311-325 (1992)); anti-mouse M2 rat hybridoma (Lagenaur & Schachner J. Supramol. Struct. Cell Biochem. 15, 335-346 (1981)); anti-BrdU mIgG$_1$ or rat polyclonal, 1:400 (Boehringer-Mannheim); anti-β-galactosidase mIgG, 1:5,000 (Promega). Species-specific secondary antibodies (1:200) were conjugated to Cy3, FITC (Jackson Immunoresearch) or Alexa 488 (1:200, Molecular Probes) and sections were counterstained with Hoechst 33342. Control slides with primary or secondary antibodies omitted were performed with each series.

Cell Quantification. Surviving BrdU positive ES cells and those double labeled for markers of differentiated neural cells were counted in 3 longitudinal sections, centered at the middle of the cord and separated by 200 μm and averaged per animal.

RESULTS

Neural differentiated mouse embryonic stem (ES) cells were transplanted into a rat spinal cord 9 days after traumatic injury. Histological analysis 2-5 weeks later revealed that transplant-derived cells survived and differentiated into

astrocytes, oligodendrocytes and neurons, and migrated up to 8 mm away from the lesion edge. Furthermore, gait analysis revealed that transplanted rats exhibited hindlimb weight support and partial hindlimb coordination not found in sham-operated controls or controls transplanted with adult mouse neocortical cells.

In the first two series of studies, thoracic spinal cord injury was induced by weight-drop (a 10 gm rod, 2.5 mm in diameter, falling 25 mm) in 22 adult female Long-Evans rats (Basso, et al., J. Neurotrauma 12,1-21 (1995); Liu, et al., J. Neurosci. 17, 5395-5406 (1997)). Nine days later, 4-/4+ stage ES cell embryoid bodies derived from the D3 line (Bain, et al., Dev. Biol. 168, 342-357 (1995)) were partially trypsinized (5 min. at 37°C, 0.25% trypsin with EDTA) and cell aggregates (total of one million cells in 5 µl ES cell medium) were transplanted into the syrinx formed at the initial site of spinal cord contusion (n=11). Sham-operated control animals were handled identically, including treatment with cyclosporine, but in place of cell transplantation they received intra-syrinx injections of culture medium alone (n=11). Beginning on the day of transplantation, all rats received cyclosporine (10 µg/kg s.q.) daily to prevent rejection. Hindlimb motor function was assessed using the Basso-Beattie-Bresnahan (BBB) Locomotor Rating Scale (Basso, et al., J. Neurotrauma 12,1-21 (1995)). In another series of 11 animals (plus 11 sham-operated controls), rats received the same transplantation procedure but using ROSA26 ES cells [a *lacZ* transgene containing, β-galactosidase (β-gal)-expressing mouse ES cell line] and animals were sacrificed 2 weeks after transplantation for histology and quantitative cell counting.

Mouse ES cell-derived cells marked genetically (using the ROSA26 β-gal-expressing line) and pre-labeled *in vitro* with BrdU (24 hr pulse, 10 µM) could be identified *in situ* 14-33 d after transplantation; alternatively identification could be achieved with the mouse specific antibodies M2 (Lagenaur & Schachner J. Supramol. Struct. Cell Biochem. 15, 335-346 (1981)), EMA (Baumrind, et al., Dev. Dyn. 194, 311-325 (1992)) or Thy 1.1/1.2 (data not shown for EMA and Thy 1.1/1.2). When examined 2-5 weeks after transplantation, ES cell-derived cells were found in aggregates or dispersed singly throughout the injury site; furthermore single cells could be found as far as 8 mm away from the syrinx edge in either the rostral or

caudal direction (Fig. 1). In the majority of the transplanted rats, by 2 weeks after transplantation ES cell-derived cells filled the space normally occupied by a syrinx in medium-treated rats. By 5 weeks, the density of ES cell-derived cells in this area was reduced and replaced with an extracellular matrix containing fibers positive for Thy 1.1/1.2 labeling. The other mouse-specific markers, M2 and EMA, offered advantages over the genetic and DNA markers (which only mark cell bodies) in that they also labeled ES cell-derived processes, which were abundant in ES cell transplanted rats, but not present in sham injected rats.

Surviving ES cell-derived cells, labeled with antibodies against mouse-specific markers or BrdU, also labeled with antibodies against markers specific for oligodendrocytes (adenomatous polyposis coli gene product, APC CC-1), astrocytes (glial fibrillary acidic protein, GFAP), and neurons (neuron-specific nuclear protein, NeuN); nuclei could be clearly identified with Hoechst 33342 staining. Most surviving ES cell-derived cells were oligodendrocytes (43 ±6% of BrdU labeled cells were O1 labeled, n = 11 rats, mean ±SEM) and astrocytes (19 ±4% were GFAP labeled), but some ES cell-derived neurons (8 ±5% were NeuN labeled) were also found in the middle of the cord. Many of the ES cell-derived oligodendrocytes were also immunoreactive for myelin-basic protein, an integral component of myelin. No evidence of tumor formation was observed.

Performance in open field locomotion was enhanced by ES cell transplantation (Fig. 2). In contrast to the inability of the sham transplantation group to support weight with their hindlimbs, the ES cell transplant group demonstrated partial weight-supported ambulation. A statistical difference in BBB scores was achieved by two weeks following transplantation (Fig. 2a). After one month, a difference of two points on the BBB scale was observed between groups: 7.9 ±0.6 for sham vehicle transplantation group, 10.0 ±0.4 for ES cell transplant group. The former score signifies a gait characterized by no hindlimb weight bearing and no coordinated hindlimb movements, whereas the latter score signifies a gait characterized by partial hindlimb weight bearing and partial hindlimb coordination.

A third experimental series examined the possibility that a rat versus mouse immune response could contribute to the observed behavioral benefit. The

transplantation of 4-/4+ ES cells (ROSA26 line) 9 days after injury was compared directly to two control groups – culture medium injection and transplantation of adult mouse neocortical cells (n = 6 per group). Immuno-histologic examination of the spinal cords 5 weeks after transplantation, using antibodies directed against microglia / macrophages (CDllb) and INF-γ, revealed that a similar degree of inflammation was present in all 3 groups. Improved locomotor function as assessed with the BBB locomotor scale (with assignments made using slow motion video) was again associated only with ES cell transplantation (Fig. 2b).

In summary, it has been demonstrated that mouse ES cell-derived cells, when transplanted into the spinal cord 9 days after weight drop injury: (1) survive for at least 5 weeks; (2) migrate at least 8 mm away from the site of transplantation; (3) differentiate into astrocytes, oligodendrocytes, neurons without forming tumors; and (4) produce improved locomotor function. Behavioral recovery similar in magnitude to that shown here has previously only been shown in acute injury models (Bernstein, et al., Exp. Neurol. 98, 633-644 (1987); Bregman, et al., Exp. Neurol. 123, 3-16 (1993); Howland, et al., Exp. Neurol. 135, 123-145 (1995); Grill, et al., J. Neurosci. 17: 5560-5572 (1997)). Factors possibly responsible for the benefits observed include enhancement of myelination, reduction of delayed oligodendrocyte death, or enhancement of host axonal regeneration, for example by providing a favorable substrate for regrowth, or by producing growth factors.

EXAMPLE 2
Method to Make a Highly Enriched Oligodendrocyte Culture

A method for producing enriched cultures of ES cell-derived oligodendrocytes has been developed and the resulting oligodendrocytes are capable of myelinating axons *in vitro* and *in vivo* after transplantation in the injured and dysmyelinated spinal cord. In the first model, localized chemical demyelination injury, without damaging passing axons, was induced in the dorsal column white matter of rats. The second model utilized myelin-deficient *shiverer* (*shi/shi*) mutant mice that lack myelin basic protein (MBP).

MATERIALS AND METHODS

Animals and Care. Homozygous (*shi/shi*) *shiverer* mice and female Long-Evans rats were obtained from Jackson (Bar Harbor, ME) and Simonsen (Gilroy, CA) labs, respectively. Interventions were in accordance with the Laboratory Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals (NIH, EHEW Pub. No. 78-23, Revised, 1978) and the Guidelines and Policies for Rodent Survival Surgery provided by the Animal Studies Committee of Washington University School of Medicine. Anesthesia was induced by ketamine/medetomidine (75:0.5 mg/kg for rats, 75:1 mg/kg for mice; i.p.) and reversed with atipamezole (1.0 mg/kg, s.q.). All animals received 1) cyclosporine (10 mg/kg, s.q.) 24 hrs prior to transplant and daily thereafter, 2) antibiotics (enrofloxacin, 2.5mg/kg, s.q.) prior to surgery and daily for 3-5 days, 3) saline (2-10 ml i.p.) post-surgery and for 2 days, and 4) nutritional supplements for 3-5 days after surgery.

ES Cell Cultures. D3 (*lacZ-*) or ROSA26 (*lacZ+*) mouse ES cells were differentiated using the 4-/4+ protocol (Bain, et al., (1995) Dev. Biol. 168, 342-357; Example 1). After 8 days *in vitro*, the 4-/4+ stage floating embryoid bodies (EBs), were partially trypsinized (5 min. at 37°C, 0.25% trypsin with EDTA) and resuspended in ES cell media (ESIM) (Bain, et al., (1995) Dev. Biol. 168, 342-357; Example 1) for transplantation or further triturated to a single cell suspension for culturing in ESIM (Bain, et al., (1995) Dev. Biol. 168, 342-357; Example 1) or modified SATO defined media with or without serum (Bottenstein & Sato, (1979) Proc. Natl. Acad. Sci. USA 76, 514-7; Raff, et al., (1983) Nature 303, 390-396).

Demyelination. Demyelination of dorsal column white matter was induced chemically in rats using characterized methods (Hall, (1972) J. Cell Sci. 10, 535-546; Blakemore, (1976) Neuropathol. Appl. Neurobiol. 2, 21-39; Waxman, et al., (1979) J. Neurol. Sci. 44, 45-53; Blakemore, & Crang, (1985) J. Neurol. Sci. 70, 207-223). After a T10 laminectomy, ethidium bromide (1 µl of 0.1 % ethidium bromide in 0.9% saline) or lysophosphatidyl choline (LPC – lysolecithin; 2 µl of 1.0% LPC in 0.9% saline) was injected at a depth of 0.5 mm in the dorsal column over a 10 min period using a stereotaxic microinjector (Stoelting) and a 30 µm tip glass pipet attached to a 5 µl Hamilton syringe. Three days later, the demyelinated areas were

transplanted with partially dissociated EBs.

Preparation of Cells for Transplantation. Pre-labeled (see Cell Tracking methods below) 4-/4+ stage EBs or oligospheres were prepared for transplantation using methods described previously (Example 1) to produce suspensions of small clusters of cells. Cell density was calculated using a hemocytometer and adjusted to 50,000 viable cells per µl.

Transplantation. Demyelination injury rats received transplants of approximately 125,000 cells from partially dissociated 4-/4+ EBs or media vehicle. A 50-100 µm tip diameter glass pipet was stereotaxically advanced 0.5 mm into the dorsal column white matter. Using a stereotaxic microinjector, 2.5 µl of the ES cell suspension or vehicle media was injected at a rate of 0.25 µl/min. The needle was left in place for 5 more min., slowly withdrawn, and the laminectomy site was covered with artificial dura. In the second model, *shiverer* (*shi/shi*) mice were transplanted with 100,000 oligosphere cells or vehicle medium (n > 6 each) at the T8 & T10 level (1 µl at 2 sites 0.35 µm below the dura).

Cell tracking and immunohistochemistry. Several methods were used to track ES cells after transplantation: (1) *lacZ* transgene, (2) bromo-deoxyuridine (BrdU) DNA labeling, (3) fluorescent cell tracker orange, and/or (4) mouse specific antibodies. ROSA26 ES cells stably expressing *lacZ* were used in all transplantation experiments (Friedman,& Soriano, (1991) Genes Dev. 5, 1513-1523). ES cells were pulse labeled with BrdU (10 µM; Boehringer-Mannheim, Indianapolis, IN) for 24 hours on the 3rd to 4th day of the 4-/4+ protocol (Example 1). In addition, partially trypsin-dissociated 4-/4+ EBs were incubated with stable fluorescent marker cell tracker orange (Molecular Probes, Eugene, OR) for 20 min, washed, incubated for another 20 min, and then washed prior to transplantation. Cell tracker orange diffuses into cells and is transformed into a fixable, membrane-impermeant form in the cytoplasm.

Mouse-specific antibodies were used to detect the mouse ES cells in the rat demyelination experiments: anti-M2 (labels mouse glia > neurons), and anti-EMA (labels mouse neurons > glia). Antibodies used to identify the oligodendrocyte lineage included: anti-NG2 for oligodendrocyte progenitors (Chemicon), anti-O4 for

immature oligodendrocytes (hybridoma), anti-O1 for mature oligodendrocytes (hybridoma), anti-MBP for terminally mature oligodendrocytes (Boehringer-Mannheim). Homozygous *shiverer* (*shi/shi*) mice are devoid of MBP and the presence of MBP(+) myelin in these animals following transplantation provided a useful marker for identification of transplanted oligodendrocytes.

Electron Microscopy. EBs and cultures were processed using standard methods (Mulvey, et al., (1998) Science 282, 1494-1497). Samples were viewed with a Hitachi S-450 Scanning Electron Microscope operated at 20 KV accelerating voltage and JEOL 100CX Transmission Electron Microscope.

RESULTS

ES cells differentiated using the 4-/4+ retinoic acid protocol (Bain, et al., (1995) Dev. Biol. 168, 342-357) produce oligodendrocytes when transplanted into injured spinal cord (Example 1). Based on this protocol, methods were developed for reliable generation of mixed cultures of oligodendrocytes, neurons and astrocytes from 4-/4+ stage EBs. EBs floated in cell clusters, many of which contained internal cysts. Ultrastructural SEM examination revealed that the cells on the surface were covered with extensive microprocesses.

Immunohistochemical studies of EBs showed limited expression of markers of differentiated neural cells and less than half of the cells were nestin positive - an early marker of neural precursors. Most cells that expressed markers of differentiated neural cells were confined to the exterior of the EBs, although a subset of cells surrounding the internal cysts were also frequently labeled with neuronal markers. Ultrastructural evidence suggested that a substantial number of EB cells exhibit features of apoptotic death. Additionally, chromatin condensation (visible in Hoechst stained nuclei), consistent with cell death, was present in 10-20% of cells.

Table 1. Expression of differentiated cells in EB's and oligospheres. Percentage of EB and oligosphere cells immunoreactive for stage specific phenotypic markers (mean ±SEM, n = 5 each).

	Cell Type	NeuN	GFAP	04	01	Nestin
Single	EB	0.06 ±0.020	0.13 ±0.011	0.38 ±0.038	0.35 ±0.052	0.08 ±0.013
	Oligosphere	0.27 ±0.044	0.06 ±0.015	0.50 ±0.094	0.54 ±0.091	0.09 ±0.091
	Ratio	4.50	0.46	1.32	1.54	1.13

Further culturing of dissociated 4-/4+ EBs in standard neural media produced mixed cultures of neurons, astrocytes and oligodendrocytes. Like primary cultures, ES-derived type-I astrocytes formed a confluent layer adherent to the bottom of the dish, other cells grew on top, and neurons grew in small clumps with large bundles of axons radiating outward. Mixed cultures grew best in SATO defined media supplemented with serum and could be maintained for at least one month. Oligodendrocyte longevity was enhanced by the presence of neurons. Adding βFGF (10 ng/ml) during the first week *in vitro* enhanced oligodendrocyte production and inhibition of cell division (10^{-5} M cytosine arabinoside), as employed in previous studies of cultured ES derived neurons (Bain, et al., (1995) Dev. Biol. 168, 342-357), limited oligodendrocyte viability.

Using immunohistochemical markers as well as scanning and transmission electron microscopy, it was observed that ES cell-derived oligodendrocytes produced myelin. Individual oligodendrocytes that myelinated multiple axons and multiple segments of single axons, could be easily identified using fluorescent antibodies directed against a component of myelin (O1) found in mature myelinating cells. After 9 DIV, axonal myelin profiles with 2-3 loosely wrapped layers were common. It is known that development of compact mature myelin profiles *in vitro* typically takes 3-6 weeks, and limited survival of neurons past 14 DIV in our mixed cultures precluded examination at later stages in the current studies. In the absence of axons, oligodendrocytes formed sheets of myelin, similar to cultures of primary oligodendrocytes.

Enriched cultures of oligodendrocytes were produced by development of an intermediate *in vitro* stage termed "oligospheres". To produce oligospheres, 4-

/4+ EBs were trypsinized and triturated, then placed in T25 flasks containing 5 ml of pre-conditioned "oligosphere media" consisting of SATO defined media, β-FGF (10 ng/ml) and PDGF (2 ng/ml). This media promoted survival and proliferation of oligodendrocytes. On the fourth day, non-adherent cells (primarily oligodendrocyte precursors) were passed into fresh oligosphere media at a 1:1 ratio. The few astrocytes generated adhered to the flask and were not passed. Over 6-8 days, the cells developed as free-floating spherical cell clusters.

Immunohistochemical studies suggested that oligospheres contained a small number of neurons, few astrocytes, a large number of immature and mature oligodendrocytes, and substantial numbers of nestin positive progenitor cells (Table 1). Plating dissociated oligospheres produced cultures comprised of 92 ±7% oligodendrocytes (n = 4), the balance neurons. One-week survival periods could be readily attained and longer-term growth was possible if cultures were fed with media conditioned by oligospheres.

To evaluate differentiation of ES cells *in vivo*, in the "injured" adult CNS, partially dissociated 4-/4+ EBs were transplanted into the dorsal column of rat spinal cord 3 days after chemical demyelination. Successful engraftment in the demyelinated region was evident in 9/10 rats when examined 1 week after transplantation, as indicated by immunostaining with anti-mouse specific antibodies, *lacZ* expression, and by an increased cell density demonstrated by Hoechst 33342 labeling in transplanted animals. In rats that received a sham vehicle transplant, axons of passage were largely spared and a paucity of Hoechst nuclear labeling was present at the demyelination site. At the lesion site of transplanted rats, ES cells differentiated primarily into oligodendrocytes (anti-APC CC-1), but not astrocytes. Enhanced GFAP reactivity was consistently observed at the lesion borders in both ES cell and vehicle medium transplanted rats, indicating the association with host reactive astrocytes. Little evidence of ES cell-derived neurons (anti-NeuN or anti-neuron specific enolase) was found in the zone of demyelination or in host tissues. Nine of the ten rats that received transplants exhibited this pattern of ES cell differentiation. Histologic evidence of acute graft rejection was present in 1/10 rats.

A second study was performed to assess the potential for ES oligodendrocytes to myelinate in the dysmyelinated adult CNS. Dissociated

oligospheres were transplanted into the thoracic spinal cord of *shiverer* (*shi/shi*) mice (>2 months old), which lack MBP, an essential component of functional myelin. Transplanted cells were tracked by pre-labeling the oligospheres with the fluorescence marker cell tracker orange or by detecting MBP expressed by transplanted cells, but absent in the host *shiverer* (*shi/shi*) mice. Two weeks after transplantation, ES cell-derived cell tracker orange(+) and MBP(+) oligodendrocytes predominated in white matter. ES oligodendrocytes conformed to the organization that oligodendrocytes normally respect in white matter: ES oligodendrocytes would align with host intrafascicular oligodendrocytes and myelinate axons. Since homozygous *shiverer* mice do not exhibit substantial MBP immunoreactivity, MBP immunoreactivity could be attributed to the transplanted ES cell-derived oligodendrocytes. In oligosphere-transplanted mice, but not sham-transplanted mice, widespread MBP immunoreactivity was evident in regions surrounding the sites of transplantation and the pattern of MBP expression was similar to that found in the normal spinal cord of mice. The longitudinal parallel arrays of MBP immunoreactivity separated by spaces occupied by axons in white matter is characteristic of axonal myelination.

The experiments described in this example demonstrate that ES cells can be used to reliably generate mixed and enriched cultures of oligodendrocytes and that these oligodendrocytes are capable of producing myelin and myelinating axons *in vitro*. In addition, transplanted ES cells can: 1) preferentially differentiate into oligodendrocytes in areas of demyelination, suggesting that environmental cues in the injury site can direct ES cell differentiation, and 2) myelinate host axons in the dysmyelinated spinal cord.

This is believed to be the first demonstration of the ability of ES cell derived oligodendrocytes to myelinate *in vitro* and to survive and myelinate axons in the mature CNS after transplantation. These findings in the mature CNS are particularly relevant since the most common disorders that are targets for therapeutic strategies of remyelination are in adults. In particular, the data demonstrate that injured demyelinated areas of the adult CNS may preferentially stimulate oligodendrocyte differentiation.

Remyelination is an enticing mechanism potentially underlying the rapid recovery of locomotor function observed when dissociated 4-/4+ stage ES cells

were transplanted 9 days after moderate contusion injury in rats (Example 1). Significant recovery of locomotion was first evident 11 days after transplantation and oligodendrocytes represented the largest differentiated population of ES cell derived cells in that study.

The above results also suggest that local conditions in the lesioned CNS can select for differentiation or survival of particular types of ES- derived neural cells. When ES cells are transplanted into a contusion- injured spinal cord, they differentiate into substantial numbers of astrocytes and oligodendrocytes arranged in specific patterns relative to one another (Example 1). In contrast, it is shown here that primary demyelinated lesions, sparing passing axons, preferentially induce ES cells to differentiate into oligodendrocytes. This observation is compatible with the previous demonstration that CNS isolated progenitors differentiate into different neuronal phenotypes based on their site of implantation in the CNS (Vicario-Abejon, et al., (1995) J. Neurosci. 15, 6351-6363; Brustle, et al., (1995) Neuron 15, 1275-1285; Shuhonen, et al., (1996) Nature 383, 624-627). No previous report has suggested that CNS context can select for differentiation of oligodendrocytes from neural progenitors.

No evidence of ES cell derived tumor formation was found in any of the above-described *in vivo* studies or in our previous spinal cord contusion transplantation series (Vicario-Abejon, et al., (1995) J. Neurosci. 15, 6351-6363). Formation of teratomas or other tumor types remains a concern in any transplantation study. A heartening feature of ES cells is that they are the only stem cells that can be proven genetically normal by generating a normal animal after implantation into blastocysts.

EXAMPLE 3
Instructions on Preparation of Oligospheres from ES Cells

This example sets forth detailed instructions on the preparation of an oligodendrocyte-enriched cell culture from ES cells. To the extent specific media, reagents and materials are mentioned, they are intended to be illustrative, not limiting, of the invention.

The process of culturing oligospheres is outlined in this example from the point where Embryoid Bodies (EBs) have been successfully created (Example 1; Bain et al, 1998). There are many stock solutions used as components of this recipe that must be created prior to beginning a particular step. Some of these must be made fresh, others can be stored and used many times. It is advisable to read through the entire protocol first, to be sure that all materials and stock solutions have been collected and/or made up prior to beginning any recipe. Each recipe outlines the process of making a particular medium or constituent that will be used in the process of making oligospheres. Further background information may be obtained from Bain et al.'s 1998 review, "Neuron-like Cells Derived in Culture From P19 Embryonal Carcinoma and Embryonic Stem Cells." This review can be found online at http://thalamus.wustl.edu/gottlieblab/gottlieb_lab_3.html.

SATO 100X Stock Recipe

Procedural Notes: SATO 100X stock solution is the medium supplement basis for SATO-no-serum medium and is the first series of steps in the process of making oligospheres. All solutes can be weighed outside the hood since the SATO 100X stock will be sterile filtered before it is aliquoted and frozen down. In the interest of time efficiency, weigh out all solutes at once, make up individual stock solutions, then combine them and filter. It is also advisable to pre-label each weighing boat and glass culture tube with the substance it will contain to assure that mistakes are not made.

1) Obtain all constituent solutes and, outside the hood, weigh them in separate pre-labeled weighing boats.
   a) Crystalline BSA 200 mg
   b) Progesterone 5 mg
   c) Putrescine 32 mg
   d) L-thyroxine 2.4 mg
   e) Tri-idothyronine 2.4 mg
   f) apo-Transferrin 180 mg

g) Sodium Selenite 4 mg

2) **Stock Progesterone Solution:** dissolve 5 mg progesterone powder into 200 µL sterile filtered 100% ethanol in a 5 mL roundbottom tube. If you have already made filtered 100% ethanol, skip to step e.

a) Obtain a 60 cc sterile syringe tube and remove the plunger.

b) Pour ~50 mL 100% ethanol into syringe tube and attach a 25 mm sterile 0.2 µm filter.

c) Push ethanol through filter into a sterile 50 mL conical tube.

d) Label tube with "Filtered 100% EtOH", the date, and your initials. (This filtered 100% EtOH can be used repeatedly if sterile technique is maintained.)

e) Carefully pour 5 mg progesterone powder into a 5 mL roundbottom tube.

f) Using a micropipette, add 200 µL sterile filtered 100% EtOH to the progesterone.

g) Cap the tube and vortex until all solute is dissolved (~2 minutes).

3) **Stock Thyroxine Solution:** dissolve 2.4 mg thyroxine powder into 300 µL sterile filtered 0.1 N NaOH in a 2nd 5 mL roundbottom tube. If sterile filtered 0.1 N NaOH, has already been made, skip to step f.

a) Obtain a 50 mL vial of 0.1 N endotoxin-free NaOH (Sigma # 210-5).

b) Obtain a 60 cc sterile syringe tube and remove the plunger.

c) Pour 50 mL 0.1 N NaOH into syringe tube and attach a 25 mm sterile 0.2 µm filter.

d) Push NaOH through filter into a sterile 50 mL conical tube.

e) Label tube with "Filtered 0.1 N NaOH" and the date. (This filtered 0.1 N NaOH can be used repeatedly if sterile technique is maintained.)

f) Carefully pour 2.4 mg thyroxine powder into a 5 mL roundbottom tube.

g) Using a micropipette, add 300 µL sterile filtered 0.1 N NaOH to the thyroxine.

h) Cap the tube and vortex until all solute is dissolved (~2 minutes).

4) **Stock Tri-idothyronine Solution**: dissolve 2.4 mg tri-idothyronine into 300

μL sterile filtered 0.1 N NaOH in a 3rd 5 mL roundbottom tube.

a) Repeat procedure from step 3, substituting tri-idothryonine for thyroxine.

5) **Stock Sodium Selenite Solution:** dissolve 4 mg sodium selenite into 100 uL sterile filtered 0.1 N NaOH, in a 15 mL conical tube, then add 10 mL DMEM.

a) Repeat procedure from steps 3 and 4, this time using 4 mg sodium selenite, 100 uL sterile filtered 0.1 N NaOH, and a 15 mL sterile conical tube.

b) Using a 10 mL serological pipette, add 10 mL DMEM to 15 mL conical tube containing sodium selenite solution.

c) Using the same pipette, triterate solution 3X to mix.

6) **Stock ITS Solution:** using a 5 mL serological pipette, add 2.5 mL DMEM to the rubber-capped commercial stock tube that the ITS came in, cap tube, and vortex until all solute is dissolved.

7) Carefully pour each of the three remaining pre-weighed constituent powders, (BSA, putrecine, apo-transferrin) into separate 50 mL sterile conical tubes.

8) Using a 10 mL serological pipette, place 6 mL DMEM into each of the three 50 mL sterile conical tubes.

9) Vortex each of the three tubes until all solute is dissolved.

10) Carefully pour the contents of each of these three 50 mL conical tubes into one 50 mL conical tube, cap and mix by hand.

11) Using separate micropipette tips, add the designated amount of each individual stock solution to the 50 mL conical tube.

a) Progesterone stock solution	5 uL
b) L-thyroxine stock solution	10 uL
c) Tri-idothyronine stock solution	10 uL
d) ITS stock solution	2 mL
e) Sodium Selenite stock solution	150 uL

6) Using a 25 mL serological pipette, gently triterate at least 3X to mix solution.

7) Obtain a 60 cc sterile syringe tube and remove the plunger

8) Pour the entire solution into the syringe tube, replace the plunger, and attach a 25 mm sterile Acrodisc 0.2 μm filter.

9) Push fluid through filter into a sterile 50 mL conical tube.

10) Using a micropipette, aliquot 400 µL of this solution into 2.0 mL sterile, RNAse-free Biostore vials with caps.

11) Label vials with "SATO 100X Stock" and the date, and store at -20°C.

12) Frozen SATO 100X Stock aliquots will keep for 6 months.

SATO Constituent Stocks Recipe

Procedural Notes: SATO Constituent stock solutions are the growth factor additives for SATO-no-serum medium and, combined, are the second series of steps in the process of making oligospheres. All solutes can be weighed outside the hood since the constituent stocks will be sterile filtered before they are aliquoted and frozen down.

In this series of steps, the practitioner will make up N-acetyl, NT-3, CNTF, Hepes, and L-glutamine stock solutions. Some stocks will keep for long periods of time, some will have to be made up fresh. Unless otherwise indicated, perform these procedures in a sterile hood. In the interest of time efficiency, weigh out all solutes at once, make up individual stock solutions, then aliquot them and filter. It is also advisable to pre-label each weighing boat and glass culture tube with the substance it will contain to assure that mistakes are not made.

Sterile Filtered dd H_20 and 0.01 M PBS pH7.4

13) Small aloquots of sterile filtered dd H_20 will be needed during the following procedures. To make these aliquots, obtain one full Kimax 1L bottle of sterile (autoclaved) dd H_20 and place it in the hood.

14) Under the hood, attach a sterile bottletop filter and vacuum tube to an empty sterile Kimax 1 L bottle.

15) Pour dd H_20 through filter, into bottle.

16) Using a 25 mL serological pipette, aloquot the entire volume of sterile filtered water into sterile 50 mL tubes.

17) Tighten caps, label tubes with "Filtered dd H_20" and the date. Store aliquots at room temperature – these will keep indefinitely and can be used repeatedly

if sterile technique is maintained.

18) Obtain 50 mL of 0.01 M PBS pH 7.4 in a sterile 50 mL conical tube, pour entire volume into a 60 cc syringe tube, attach a sterile 25 mm Acrodisc 0.2 μm filter, and push fluid through filter and into a sterile 50 mL conical tube.

19) Tighten cap, label tube with "Sterile PBS" and the date. Store at room temperature – this solution will keep for a long time and the same aliquot can be used repeatedly if proper sterile technique is maintained.

20) Obtain two 50 mL aliquots of sterile water and place in the hood.

21) Using a 1 mL serological pipette, take 1 mL of sterile water from one of the aliquots and place it in a 5 mL roundbottom tube.

22) Weigh 6.3 mg N-acetyl-cysteine outside of the hood.

23) Carefully pour powder into the 1 mL volume of sterile water in the 5 mL Falcon tube from step 7.

24) Vortex until all powder is dissolved (this will take about 2 minutes).

25) In the hood, and using a 3 cc syringe tube, suck up NAC solution, attach a sterile 13 mm Acrodisc 0.2 μm filter (Sterile Acrodisc 13; 0.2 μm; single use; low protein binding; non-pyrogenic filter – Gelman Sciences # 4454) and push fluid through filter and into another sterile 5 mL Falcon tube.

26) Label tube "NAC stock" and leave in hood – this solution does not keep and will have to be made up fresh each time it is used.

27) 100X NAC stock is now ready

28) Obtain a 50 mL aloquot of sterile 0.01 M PBS and place it in the hood.

29) Using a 10 mL serological pipette, transfer 10 mL sterile 0.01 M PBS into a sterile 15 mL centrifuge tube.

30) Cap the tube and bring it to the scale.

31) Weigh 0.1 g BSA and carefully pour into the 15 mL tube from the last step

32) Cap tube tightly and vortex for 30 seconds

33) Obtain a sterile 10 cc syringe tube, attach a sterile 13 mm Acrodisc 0.2 μm filter, remove the plunger, pour BSA solution into syringe body, replace plunger, and push fluid volume through filter into another sterile 15 mL conical tube - this completes the preparation of a 1% BSA stock solution.

34) Under the hood, and using a 1 mL serological pipette, aliquot 1 mL 1% BSA

stock solution into each of ten 2 mL Biostore vials.

35) Cap the vials tightly, label with "1% BSA" and the date.

36) Store 1% BSA stock at -20°C – this solution will keep for ~6 months.

37) Obtain concentrated NT-3 stock solution (gift) and five to ten 1 mL aliquots of the 1% BSA in PBS stock solution (the number needed depends on how much concentrated NT-3 stock is available).

38) Thaw 1% BSA aliquots at room temperature for 5 minutes.

39) Using a micropipette, transfer 900 µL of the 1% BSA stock and 100 µL of the concentrated NT-3 stock into a new 2 mL Biostore vial.

40) Repeat this process until there is no more concentrated NT-3 stock left.

41) Cap vials tightly and label with "100µg/mL NT-3" and the date.

42) Store diluted NT-3 stock at -20°C – this stock will keep for ~6 months.

43) Obtain a 10 µg vial of CNTF powder (it comes in these quantities directly from Sigma) and a 1 mL aliquot of the 1% BSA stock.

44) Let the 1% BSA stock thaw at room temperature for 5 minutes.

45) Under the hood, using a micropipette, add 400 µL of 1% BSA stock to the 10 µg vial of CNTF (it comes pre-measured from the company).

46) Vortex solution for ~1 minute or until all powder is dissolved.

47) CNTF stock is now ready. This stock is usually made fresh each time we need it, but many aliquots can be made up at one time and stored at -20°C for several months.

48) Obtain a 50 mL aliquot of sterile ddI water.

49) Weigh out 11.915 g Hepes powder and carefully pour into an empty, sterile 50 mL conical tube.

50) Pour ddI water from aliquot into Hepes tube up to 50 mL mark.

51) Vortex until all powder is dissolved. This may take upwards of 30 minutes.

52) Fill remaining volume in tube up to 50 mL with ddI water.

53) Vortex 1 minute.

54) Obtain a sterile 60 cc syringe tube, attach a sterile 25 mm Acrodisc 0.2 µm filter, remove the plunger, pour Hepes solution into syringe body, replace plunger, and push fluid volume through filter into another sterile 50 mL conical tube - this completes the preparation of Hepes stock solution.

55) Cap tube tightly and label with "Hepes Stock"and the date.

56) Store at room temperature – this aliquot can be used repeatedly if sterile technique is maintained and will keep on the shelf for ~4 months.

57) Obtain one 200 mM L-glutamine vial.

58) Obtain one 50 mL aliquot of sterile ddI water.

59) Under the hood, fill a 60 cc sterile syringe, with a 21 gauge needle attached, with the 50 mL sterile water from the last step.

60) Carefully remove aluminum cap tab and slowly inject 50 cc ddI water into bottle.

61) Give the bottle two or three shakes to remove any residual powder from top and sides of bottle.

62) Carefully remove the aluminum and rubber stopper cap from the bottle – this must be accomplished without losing any powder or liquid. Save the stopper in the sterile hood.

63) Cover the bottle opening with parafilm and incubate in a 37°C waterbath for 5 minutes.

64) Under the hood, replace the rubber stopper cap and shake until all powder is dissolved – solution should appear completely clear and colorless.

65) Obtain a 60 cc sterile syringe tube, attach a sterile 25 mm Acrodisc 0.2 µm filter, remove the plunger, pour L-glutamine solution into syringe body, replace plunger, and push fluid volume through filter into a sterile 50 mL conical tube - this completes the preparation of L-glutamine stock solution.

66) Using a micropipette, aliquot 500 µL L-glutamine stock solution into 0.75 mL Biostore vials.

67) Tighten caps and label each vial with "200 mM L-glut" and the date.

68) Store at -20°C – this stock solution will keep for ~ 3 months and needs to be protected from light exposure.

69) Immediately before use, this stock must be thawed in a 37°C water bath for 5 minutes and vortexed to redissolve any solute that has fallen out of solution during storage.

Embryonic Stem Cell Induction Medium (ESIM) Recipe

Procedural Notes: As with MS media, ESIM is made in 1L bottles without use of volumetrics for exact measurement of final volumes. For this reason, it is important to be accurate in measuring volumes of components and consistent in the type of media bottle. Again, it is important to keep sterile technique always in mind when making ESIM. It is advisable to weigh out all powders at once, outside the hood, and label each weighing boat and tube with the substance it will contain.

72) Obtain 100 mL NCS (New Calf Serum) and 100 mL FBS (Fetal Bovine Serum).

73) Place serums in a 60°C water bath for 30 minutes to heat-inactivate the compliments in the serum.

74) During this water bath inactivation time, make up NS (Nucleoside Stock).

75) To make NS: weigh out all constituent nucleoside powders at one time outside the hood.

a) Adenosine	40 mg
b) Guanosine	42.5 mg
c) Citidine	36.5 mg
d) Uridine	36.5 mg
e) Thymidine	12 mg

76) Carefully pour all pre-weighed nucleoside powders into a sterile 50 mL conical tube.

77) Pour 50 mL sterile dd H_20 into the 50 mL tube containing the nucleoside powders. This quantity of stock is enough to make 5 L ESIM and can be used repeatedly if sterile technique is maintained.

78) Place solution into a 37°C water bath for ~15 minutes, shaking intermittently until solutes are completely dissolved.

79) Pour entire solution into a sterile 60 cc syringe tube, replace the plunger, and attach a 25 mm sterile Acrodisc 0.2 μm filter. Push solution through filter into another sterile 50 mL conical tube.

80) Label with "NS" and the date. This stock will keep for 6 months and can be

used repeatedly if sterile technique is maintained – however, before each use of the NS, repeat step 7.

81) Obtain one of the sterilized empty Kimax 1L bottles made during the MS recipe.

82) Obtain 1L DMEM and pour 790 mL into the empty sterile 1L Kimax bottle.

83) Pour heat-inactivated serums (NCS, FBS) directly into the 1L Kimax containing 790 mL DMEM.

84) Using a 10 mL seriological pipette, add 10 mL NS stock to the DMEM plus serum solution.

85) Shake bottle by hand to mix solution.

86) Attach a 500 mL bottletop filter and vacuum tube to another sterile, empty Kimax 1L bottle.

87) Pour ESIM solution through filter into the second bottle.

88) Cap new bottle containing medium tightly, label with "ESIM", the date, your initials, and store in 4°C refrigerator.

89) ESIM is now ready for use, will keep for ~30 days, and can be used repeatedly if sterile technique is maintained.

SATO-no-serum Medium Recipe

Procedural Notes: This procedure is the final recipe for creating SATO-no-serum medium, the culturing medium used in creating oligospheres from EBs.

91) Obtain all constituents listed in the SATO-serum free Media Constituents Table from their various storage locations.

92) Under the hood, using a 25 mL serological pipette, transfer 37 mL DMEM into a sterile 50 mL conical tube.

93) Using a micropipette, transfer 400 µL of 100 mM MEM Sodium Pyruvate stock into DMEM solution from the last step.

94) Using a micropipette, transfer 400 µL of the SATO 100X stock into DMEM solution from the last step.

95) Using a micropipette, transfer 400 µL of the N-acetyl-cysteine stock into

DMEM solution from the last step.

96) Using a micropipette, transfer 20 µL of the NT-3 stock into DMEM solution from the last step.

97) Using a micropipette, transfer 32 µL of the CNTF stock into DMEM solution from the last step.

98) Using a micropipette, transfer 600 µL of the HEPES stock into DMEM solution from the last step.

99) Using a micropipette, transfer 400 µL of the L-glutamine stock into DMEM solution from the last step.

100) Using a 25 mL serological pipette, triturate 3X to mix solution.

101) Obtain a sterile 60 cc syringe, attach a sterile Acrodisc filter, remove the plunger, pour SATO-no-serum solution into syringe body, replace plunger, and push fluid volume through filter into another sterile 50 mL conical tube

102) Label tube with "SATO-no-serum", the date, and your initials.

103) Store at 4°C – this medium will keep for ~ 14 days and can be used repeatedly during this time if sterile technique is maintained.

Oligosphere Recipe

Procedural Notes: It is very important to carry out all procedures in a sterile hood and with sterile instruments and tubes unless otherwise indicated. Specifically, it is advisable to use a fresh, sterile, serological pipette for each step unless repeated use is indicated. Centrifugation is not generally performed in a hood, but tube caps must be kept tight during this procedure to insure that cells remain in a sterile environment. Do not allow any liquids or pipettes to contact the necks of flasks at any time because this part of the flask can be contaminated. To prevent any medium from contacting the neck when handling cells in T25 flasks, keep flasks nearly level with a very slight tilt toward the rear of the flask.

Finally, it is important to loosen caps on T25 flasks when cells are placed in an incubator. This will ensure that the atmosphere of the incubator can equilibrate with

the gases inside the flask. Conversely, caps should be tightened on flasks when they are removed from the incubator to ensure that a sterile environment is maintained.

Gelatin-coated Flasks

1) At least 6 hours before beginning the oligosphere procedure, obtain ten T25 flasks for gelatin coating procedure.
2) Obtain 2% sterile gelatin solution from 4°C refrigerator and let sit under the hood until solution is room temperature, clear, and homogenous.
3) Under the hood, using a 25 mL serological pipette, transfer 22.5 mL sterile dd (double-deionized) H_20 into a 50 mL conical tube.
4) Using a 5 mL serological pipette, add 2.5 mL 2% sterile gelatin solution. This makes a 0.2% (1:9 dilution) gelatin solution.
5) Using a 25 mL serological pipette, triturate solution 3X to mix.
6) Using a 5 mL serological pipette, add 5 mL 0.2% gelatin solution to each T25 flask.
7) Tighten cap and let sit at room temperature for at least 6 hours – for optimal gelatin bonding, overnight setting is preferred. Gelatin-coated flasks will keep at room temperature for several weeks, so these flasks can be used for other procedures as well.
8) Remember: immediately before using any of the gelatin-coated flasks, remove cap and tilt flask to allow liquid to run to one side. Suction off all liquid, being careful not to scrape gelatin coated bottom.

Final SATO-no-serum Medium Preparation

9) Obtain a SATO-no-serum medium (see SATO-no-serum Medium Recipe) aliquot from the 4°C refrigerator and let sit under the hood until solution is room temperature (~ 15 min.).
10) Under the hood, using a 10 mL serological pipette, transfer 10 mL SATO-

no-serum medium to a 15 mL conical tube.

11) Place remaining SATO-no-serum medium aliquot back in 4°C refrigerator.

12) Using a micropipette, add 1 µL 100µg/mL bFGF stock to the 10 mL SATO-no-serum medium in the 15 mL conical tube.

13) Using a micropipette, add 2 µL 10µg/mL PDGF stock to SATO-no-serum plus bFGF solution.

14) Using a 10 mL serological pipette, triturate 3X to mix solution (this completes the preparation of the SATO-no-serum plus bFGF and PDGF stock solution for steps 28 and 31 – this must be made up fresh before each use). This quantity of stock will be enough to perform the first day's oligosphere procedure on 1 dish of EBs.

Dissociating EBs

15) Obtain MS (Media Stock) and ESIM (Embryonic Stem Cell Induction Medium) from the 4°C refrigerator and let them sit under the hood until solutions are room temperature (~ 15 min.).

16) Under the hood, using a 10 mL serological pipette, transfer EBs (4-/4+), along with all their media, from the 100 mm Canada petri dish (this is what the EBs are grown in) into a 15 mL conical tube.

17) Let EBs settle by gravity to bottom of tube for 5 minutes.

18) Suction off supernatant media to just above EB layer (be careful not to suction off the EBs).

19) Using a 10 mL serological pipette, add 10 mL of MS to settled EBs (this is used to dilute any depleted media that remains with the cells).

20) Repeat steps 17 and 18.

21) Using a 5 mL serological pipette, add 2 mL of 0.25% Trypsin containing EDTA to the 15 mL tube.

22) Incubate for 6-8 minutes in a 5% CO_2 atmosphere 37°C incubator (every two minutes the suspension should be gently agitated by hand to aid in dissociation).

23) Using a 10 mL serological pipette, add 10 mL ESIM (ES Cell Induction Medium) to the trypsin and cell suspension (this is used to stop trypsin activity at a specific time point).

24) Place MS and ESIM bottles back in the 4°C refrigerator.

25) Centrifuge for 5 minutes at 850 G.

26) While the centrifuge is running, suction off liquid from two T25 gelatin-coated flasks (careful not to scrape gel-coating off the bottom of the flask). See step 8.

27) Remove cells from centrifuge and, under the hood, suction off supernatant media to just above cell layer.

28) Using a 5 mL serological pipette, add 2 mL SATO-no-serum medium with bFGF and PDGF (see steps 9-14) to the cells in the 15 mL conical tube.

29) Gently stir a micropipette tip through the cell-slurry. Naked DNA from dead cells should stick to the end of this pipette tip and can be lifted directed out of the cell slurry, then discarded.

30) Using a cotton-plugged, borosilicate glass Pasteur pipette with a rubber bulb attached, triturate 10x to dissociate EBs into single-cell suspension.

Using a 10 mL serological pipette, add 8 mL SATO-no-serum medium with bFGF and PDGF

Oligosphere Materials Table

		Manufacturing		Distributing	
Product Name	Size	Company	Product No.	Company	Cat. No.
Falcon Conical Tube	50 mL	Becton-Dickinson	2070	Fisher	14-432-22
	15 mL	Becton-Dickinson	2095	Fisher	05-527-90
Roundbottom Tube	5 mL	Becton-Dickinson	2054	Fisher	14-956-1B
Serological Pipette	50 mL	Becton-Dickinson	7550	Fisher	13-675-27
	25 mL	Becton-Dickinson	7525	Fisher	13-668-2
	10 mL	Becton-Dickinson	7551	Fisher	13-675-20
	5 mL	Becton-Dickinson	7543	Fisher	13-675-22
	1 mL	Becton-Dickinson	7521	Fisher	13-675-15B

Biostore Vials (Sterile, RNAse Free)	2 mL	United Laboratory Plastics	UP 2231	-------	-------
	0.75 mL		UP 2235	-------	-------
T25 Culture Flask	125 mL	Corning Costar	3055	Fisher	07-200-72
Type B 2% Gelatin	100 mL	Sigma	G-1393	-------	-------
0.25% Trypsin-EDTA	100 mL	GibcoBRL	25200-056	-------	-------
Pasteur pipette (borosilicate glass)	9 mL	Fisher	13-678-8B	-------	-------
Syringe Tube	60 cc	Becton-Dickinson	309663	Fisher	14-823-2D
	10 cc	Becton-Dickinson	309604	Fisher	14-823-2A
	3 cc	Becton-Dickinson	309585	Fisher	14-829-40
Syringe Needle	21 gauge	Becton-Dickinson	305165	Fisher	14-826C
Bottletop Filter 37T (PES membrane)	500 mL	Nalgene	295-3320	Fisher	09-740-
Acrodisc Filter (0.2 micron)	25 mm	Pall Gelman	4192	VWR	28144-040
	13 mm	Pall Gelman	4454	VWR	28142-340
Kimax Media Bottle	1 L	Kimble	61110-1000	Fisher	06-421-4
Polypropylene Cap For 1L Kimax Bottle (Bonded PTFE liner)	1 L	Kimble	73808-33430	Fisher	03-340-30

The present invention is not limited to the embodiments described and exemplified above, but is capable of variation and modification without departure from the scope of the appended claims.

What is claimed:

1. A method to make an oligodendrocyte-enriched cell culture, comprising culturing pleuripotent vertebrate cells in at least 50% preconditioned oligodendrocyte culture medium.

2. The method of claim 1, in which the pleuripotent cells are ES cells.

3. The method of claim 2, in which the cells have been cultured to obtain 4-/4+ stage embryoid bodies.

4. The method of claim 1, in which the pleuripotent vertebrate cells are obtained from a vertebrate selected from the group consisting of mouse, rat, hamster, dog, cat, monkey and human.

5. The method of claim 2, in which the cells have been treated with retinoic acid.

6. An *in vitro* differentiated culture of neural cells, which is comprised of at least 20% immature and mature oligodendrocytes.

7. The culture of claim 6, comprising at least 50% immature and mature oligodendrocytes.

8. The culture of claim 6, wherein the cells are from a vertebrate selected from the group consisting of mouse, rat, hamster, dog, cat, monkey and human.

9. The culture of claim 6, which is made by a method comprising culturing pleuripotent vertebrate cells in at least 50% preconditioned oligodendrocyte culture medium.

10. A method to treat spinal cord degeneration in patients in need of such treatment, comprising the steps of:

a) transplanting *in vitro* differentiated neural cells into the spinal cord of the patient at the site of degeneration; and

b) allowing the transplanted cells to grow, the growth of the cells providing amelioration or reversal of the spinal cord degeneration.

11. The method of claim 10, in which the *in vitro* differentiated neural cells comprise at least 20% immature and mature oligodendrocytes.

12. The method of claim 10, in which the neural cells are prepared by culturing pleuripotent vertebrate cells in at least 50% preconditioned oligodendrocyte culture medium.

13. A method of making an oligodendrocyte-enriched cell culture from ES cells, comprising the steps of:

a) providing embryoid bodies from the ES cells;

b) dissociating the embryoid bodies to produce dissociated cells;

c) culturing the dissociated cells in modified SATO medium;

d) shaking flasks in which the dissociated cells are cultured to suspend loosely adhering cells, primarily comprising oligodendrocytes;

e) transferring an aliquot of the suspended cells to a new flask containing an approximately equivalent aliquot of modified SATO medium; and

f) culturing the transferred cells, thereby producing the oligodendrocyte-enriched cell culture.

14. An oligodendrocyte-enriched cell culture produced by the method of claim 13.

Fig. 1

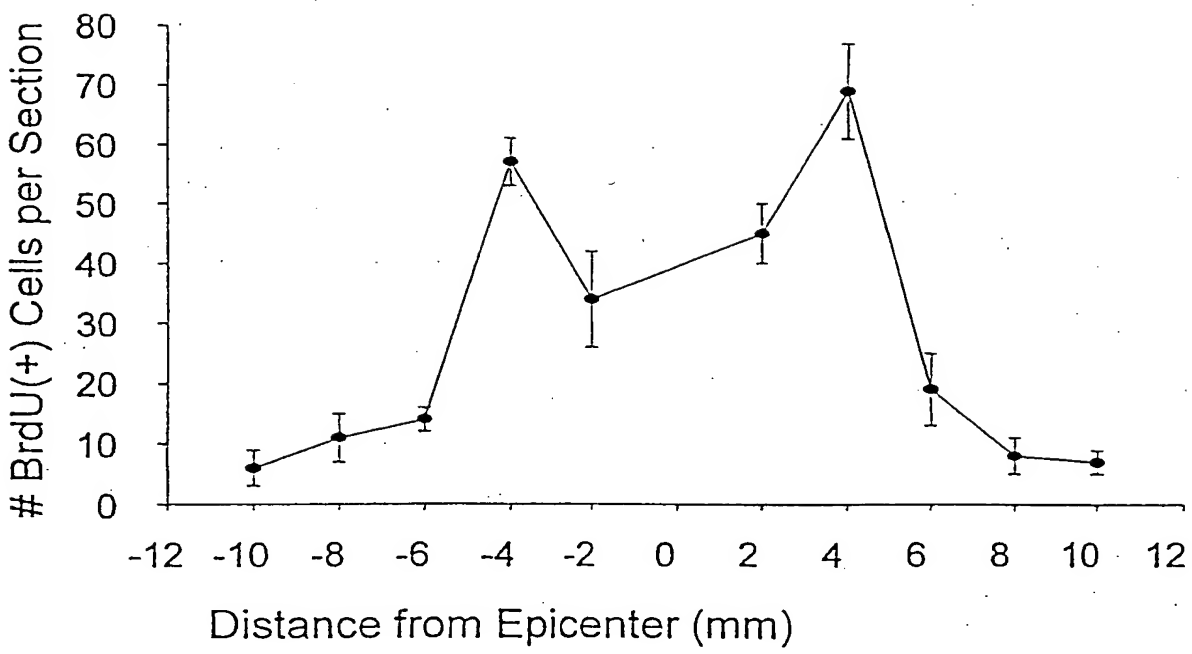


Fig. 2a

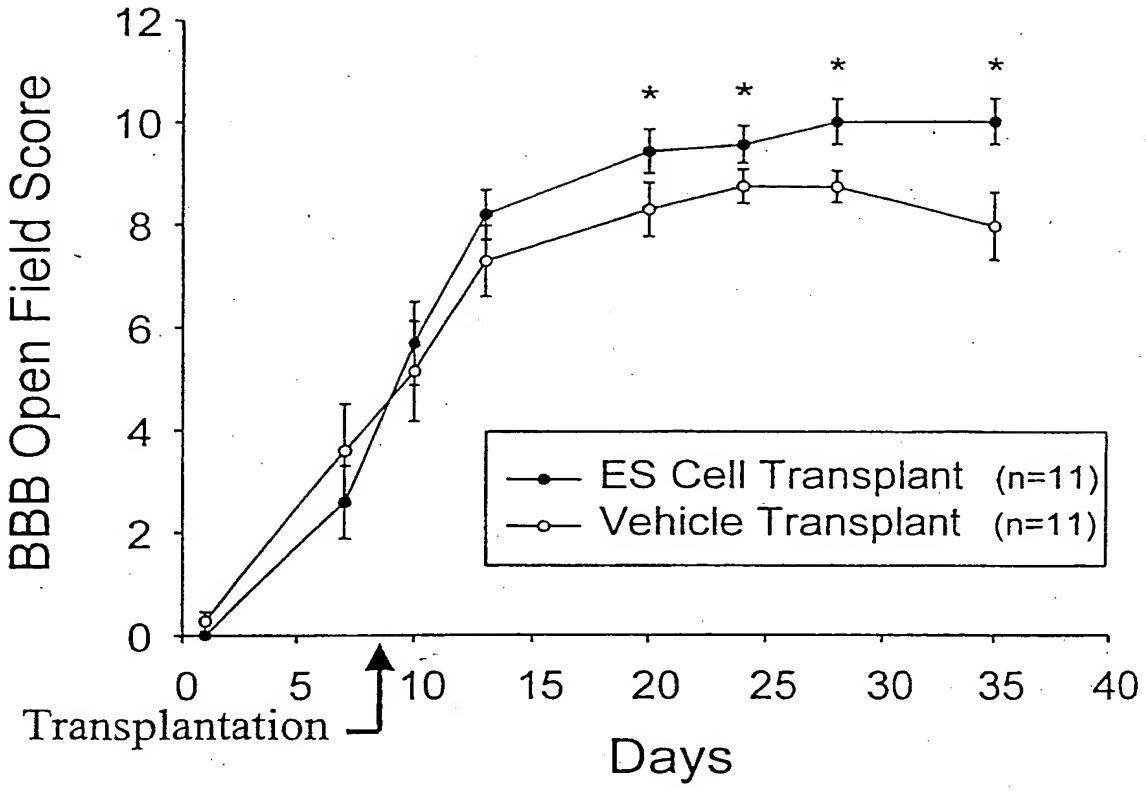
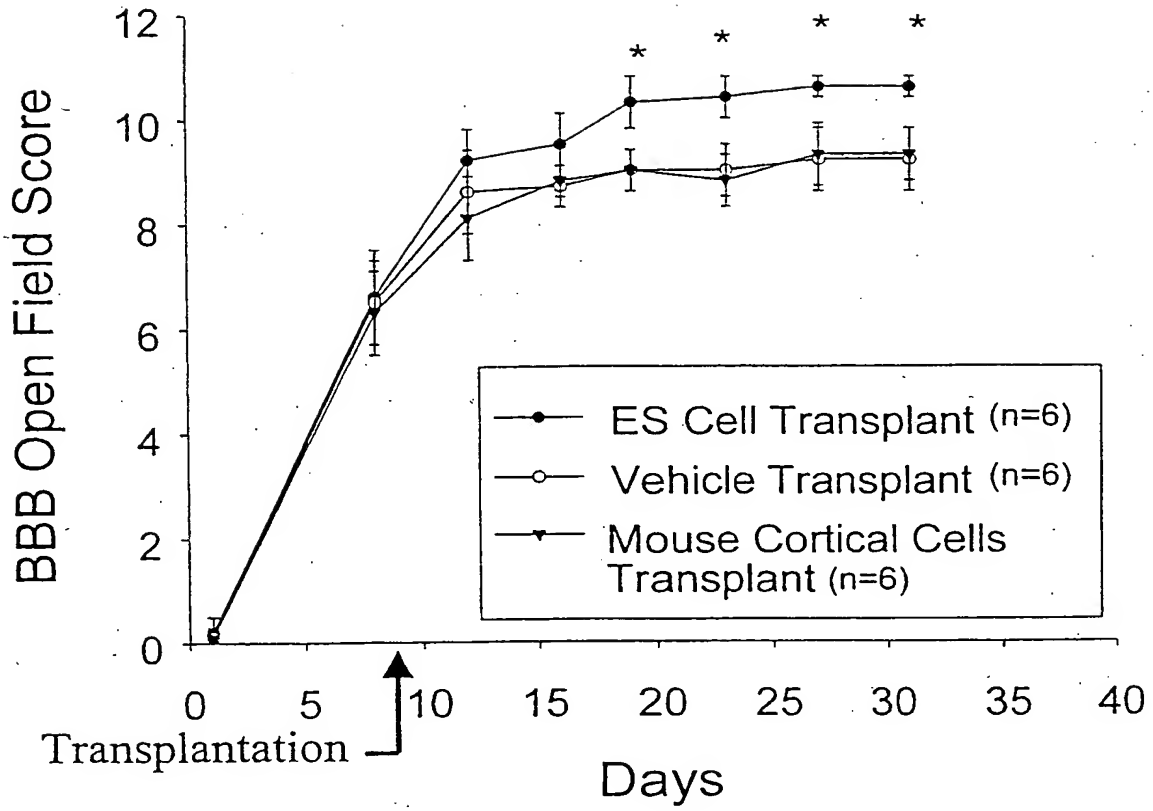


Fig. 2b



INTERNATIONAL SEARCH REPORT

Internati... ...l application No.
PCT/US00/41367

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A01N 63/00, 65/00; C12P 21/04.
US CL : 424/93.1, 93.21; 435/70.1.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 424/93.1, 93.21; 435/70.1.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WEST, STN

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MUJTABA, T., Lineage-Restricted Neural Precursors can be isolated from both the mouse neural tube and cultured ES cells, Developmental Biology 214, 113-127, 01 October 1999.	1-9, 13, 14.
Y	BRUSTLE, O., Embryonic Stem Cell-Derived Glial Precursors: A Source of Myelinating transplants, 30 July 1999, Science Vol. 285, page 754-756.	1-9, 13 and 14.
Y — P	LEE, J.C. et. al., Gliogenesis in the Central Nervous System. Glia, Vol. 31(2), pages 105-121, April 2000.	1-9, 13 and 14.

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search	Date of mailing of the international search report
	12 MAR 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Eleanor Sorbello Telephone No. 703-308-0196

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INTERNATIONAL SEARCH REPORT

Internati. application No.

PCT/US00/41367

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, A	LIU, S. et al., Embryonic stem cells differentiate into oligodendrocytes and myelinate in culture and after spinal cord transplantation..PNAS,Vol.97,no.11, 23 May 2000,pages 6126-6131.	1-9, 13 and 14
Y, P	MCDONALD, J.W. et al., Transplanted embryonic stem cells survive, differentiate and promote recovery in injured rat spinal cord., Nature Medicine, December 1999, Vol. 5., NO: 12, pages 1410-1412	1-9, 13, 14
Y	RAO, M., Multipotent and restricted precursors in the central nervous system 15 August 1999. The Anatomical record, Vol. 257, pages 137-148.	1-9, 13 and 14
Y	US 5,750,103 A(CHERKSEY) 12 May 1998 (12.05.1998), see abstract.	10-12

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